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GENETICS AND PLANT BREEDING

Use of proximal hypocotyl segment for high-through put transgenic development of tomato

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Abstract

Achieving high-throughput and efficientregenerationare the top priorities of any Agrobacterium mediated plant transformation experiments to develop large numbers of in vitrotransformants. The type of explant plays a critical role in shoot regeneration efficiency. In the present investigation, an attempt was made tostudy the effect of various segments of hypocotyl and abaxial/adaxial orientation of cotyledon explants on regeneration efficiency in tomato.A plant transformation vector,pGRNAi-harboringdsRNAexpressing constructstargeted totwo genes of Helicoverpaarmigera,serine proteaseand chymotrypsinindependently wereused totransformtomato.Of the three segments of hypocotylsobtained from 12 day old seedlings, the proximal (closest to shoot apex) segment hadyielded highest regeneration (28.65%)compared to the middle (11.86%)and the distal segments (11.20%).In cotyledon explants,those incubated with their abaxial surface in contact with media exhibited highest regeneration (20.83%) compared to adaxially placed cotyledon explants. However, of the two-explant types, hypocotyls had higher regeneration compared to cotyledons. The molecular characterization of putative transformants through PCR and Southern blot analysis revealed the presence of the transgene. Thus, these results will aid in obtaining high-throughput regeneration in transformation of tomato in particular and other crops in general.

Highlights

- Best explant for tomato transformation is the proximal portion of hypocotyl where highest regeneration efficiency was obtained.
- Abaxial incubation of cotyledon yielded higher regeneration than adaxial.

Keywords: Cotyledon, Hypocotyl, PCR, regeneration efficiency, Southern blotting

Tomato (Solanum lycopersicumL.) is a globally cultivated vegetable croprankingsecond after potato and itbelongs to the familySolanaceae. Due to its high nutritional value, it is extensively consumedas fresh, cooked as well asin processedform. It is rich in antioxidants, carotenoids, lycopene and other nutrients. Every year around 150 million metric tons of tomatoes were producedglobally (FAO 2012). In India, its productivitywas 19.9 metric tons per hectare during 2010.With the advent of the next generation

sequencing, massive sequence information ismade available in a number of crops, thus,necessitating the use of high-throughput functional validation tools such as RNA interference, which in turn require high-throughput plant transformationprotocols. Plant transformation is also an important tool in genetic enhancement of crops including the traits such as tolerance to biotic and abiotic stresses and improved post-harvest as well as agronomic traits.

Employing the recent advancements in the field of molecular biology such as RNA interferenceand site directed genome editing tools such as ZFNs (zinc finger nucleases) and CRISPR/cas9 (clustered regularly interspersed short palindromic repeat/ CRISPR associated protein 9)and various functional genes have been elucidated and metabolic pathways have been traced by gene silencing or bythe down regulation of target genes. Utilizing Agrobacterium mediated gene transformation and RNAi approach,today a number of gene functions in various crop plants has been efficiently characterized (Xiong*et al.,* 2013;Zhu *et al.,* 2012). Furthermore, traits of interest like herbicide tolerance, nutritional enhancement and bio-fortification by the accumulation of folic acid, anthocyanin enhancement (Butelli *et al.,* 2008; Maligeppagol *et al.,* 2013), delayed ripening of fruit (Gupta *et al.,* 2013; Klee *et al.,* 1991), drought, salinity and heat tolerance traits (Shaha *et al.,* 2013; Mahesh *et al.,* 2013) have been engineered.

Among the Solanaceouscrops tomato has been widely used in the Agrobacterium mediated transformation experiments for the trait developmentdue to the rationale that it has small genome size, amenability for genetic manipulation, availability of the annotatedgenome sequence, (Sato *et al.,* 2012) etc., because of these facts today tomato has been considered asamodel plant next to tobacco. As a result, it has been usedextensively to studythe fruit development and ripening, furthermore it isused to help unraveling the mysteriesin functional genomics, proteomics, and metabolomics. After McCormick's (1986) first report on Agrobacterium mediated tomato transformation, today a number of papers have been published, describing the regeneration and transformation efficiency, with various parameters, like, type of the hormone concentration, selection medium, cocultivation method, time duration of cocultivation, genotype, age and type of the explants etc., (Devi *et al.,* 2013; Sharma *et al.,* 2012). Among these parameters, explant plays an important role in determining the regeneration efficiency, choice of the explant and the orientation of incubation in the culture medium.i.e., polarity of hypocotyls and orientation of cotyledon determines the success of regeneration (Sharma *et al.,* 2009; Bhatia *et al.,* 2005; George, 1993). So far, explants such as, leaf, root, hypocotyl, cotyledon and petiole have been used for tomato regeneration, (Sharma and Srivastava, 2014) and different regeneration responseswere obtained. However, for the Agrobacterium mediated transformation in tomato both hypocotyl and cotyledon have been widely used. One of the bottlenecks in the Agrobacterium mediated tomato regeneration is the reproducibility of the protocols among the various cultivars/genotypes used for transformation (Sharma *et al.,* 2009). Hence, it is required to optimize each genotype before it is beingused for Agrobacterium mediated tomato transformation for the trait development.

In the present study, a good general tomato combiner Arkavikas (Bhatt *et al.,* 2001)was used for transformation. Very few reports are available on tomato cultivar Arkavikasfor shoot regeneration and transgenic development (Manamohan *et al*., 2011; Shivakumar *et al*., 2007; Sharma *et al*., 2009)and to provide the maximum information to utilize this cultivarfor the transgenic development an effort has been done. In our previous report, we optimized the protocol to obtain high shoot regenerationpercentage using pCAMBIA2301 vector carrying a *dreb*1A gene with hypocotyl as explant (Manamohan *et al*., 2011). In this report it was extended to assess the distinct shoot regeneration efficiency amongthe hypocotyl segments and also examined the influence of the cotyledon orientationon shoot regeneration by employing amodified in-house built binary vector pGRNAi (Manamohan *et al*., 2013) harboring the *HaSP* and *HaCHY* gene.

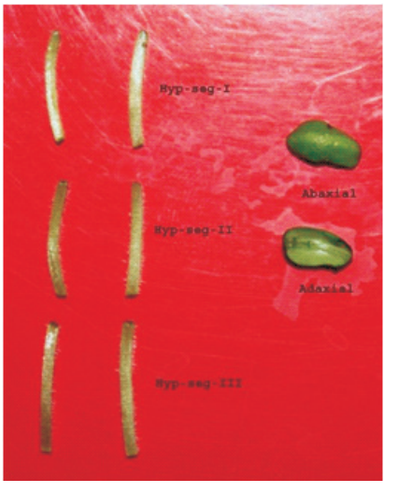

Fig.1. Preparation of the explants. The hypocotyl was dissected into three segments and cotyledon orientation.

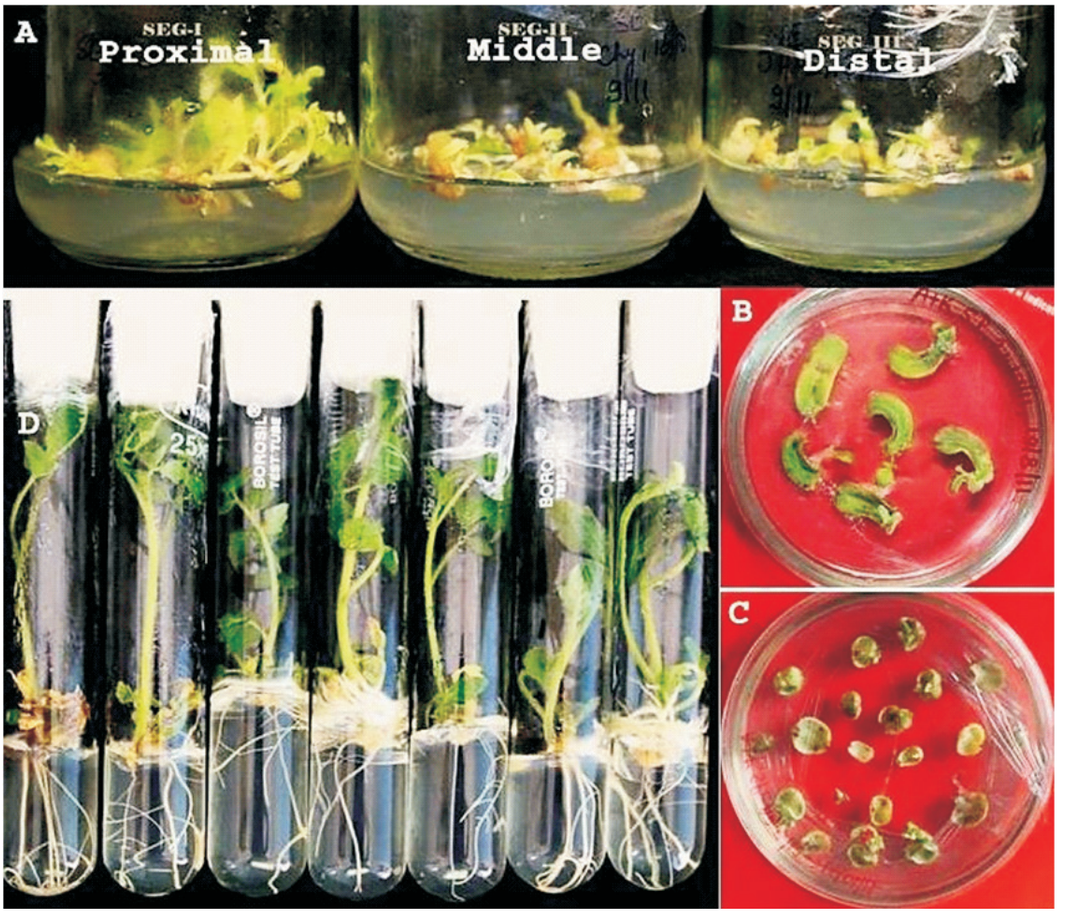

Figure 2. Regeneration response of hypocotyl and cotyledon explants. (A) Different hypocotyl type segments displayed a regeneration response in antibiotic selection medium. (B) Adaxial and (C) abaxial orientation of the cotyledon in the MS medium and its regeneration response (D) The rooting of the excised shoots in the rooting medium.

Materials and Methods

Preparation of Tissue culture medium

For the *Agrobacterium* mediated transformation of tomato, the basal medium of Murashige and Skoog (1962) wasused. Different types of hormone combination for the seed germination, shoot induction, shoot elongation, and root induction were mentioned in the Table 1. All the MS medium components were adjusted to pH 5.8 prior to the addition of 0.3% plant tissue-culture grade agar-agar (Sigma, USA), after that all the components wereautoclaved at 121°C for 20 min. The growth regulators 6-benzylaminopurine (BAP) and Indole-3-acetic acid (IAA) were obtained from sigma, 1 mg/ml stock concentration of growth hormones were prepared using a small amount 1NNaOH. The antibiotics stock kanamycin (100mg/ml) and augmentin (300mg/ml) were prepared in Milli Q water and were stored in -20°C. Antibiotics and growth hormones were added to the autoclaved MS medium after it cooled to ~50°C.

Seed germination

Tomato cv. ArkaVikas seeds were obtained from the Division of Vegetable Crops, Indian Institute of Horticultural Research (IIHR), Bengaluru. Primarily the seeds were surface sterilized by immersing in 70% v/v ethanol for seven min, immediately seeds were

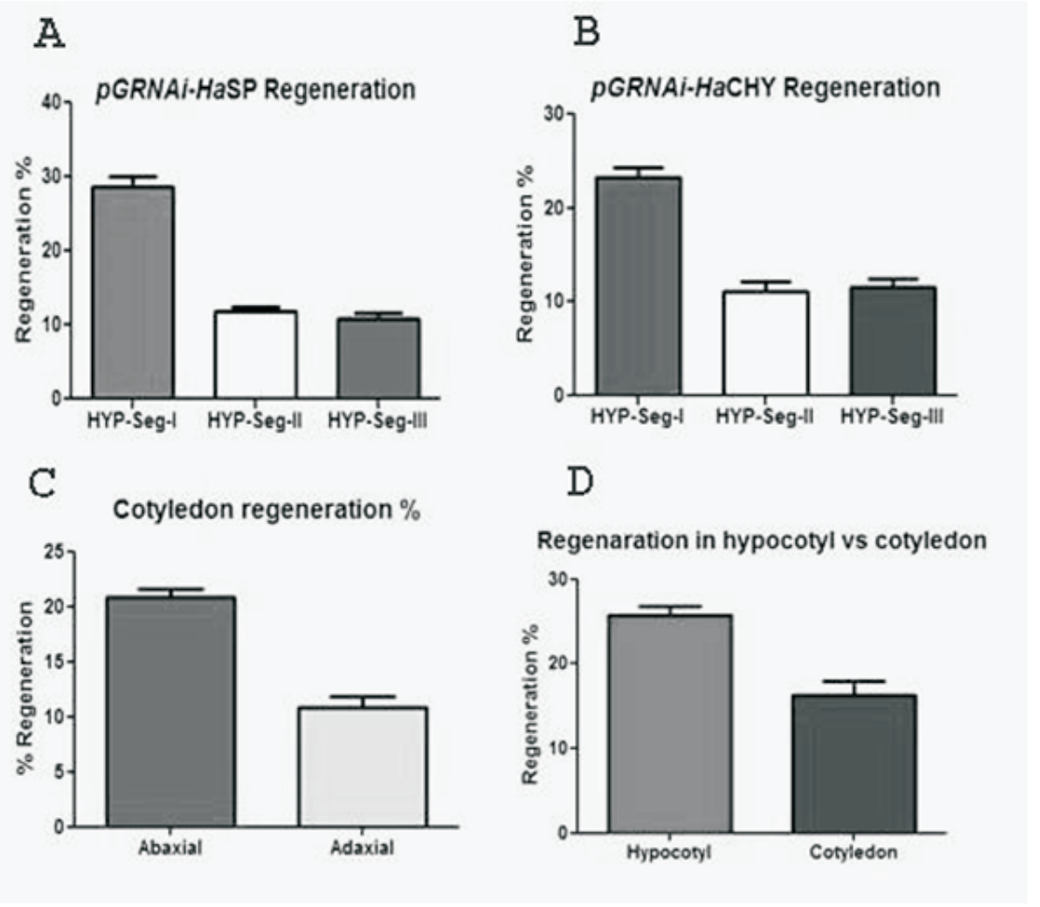

Fig.3. Statistical analysis of the shoot regeneration efficiency of the hypocotyl type explants using‘t’ test. A) Shoot regeneration efficiency was recorded highest for Hyp-seg-I compared to other two type hypocotyl segments in two vector transformed and was statistically significant ($p>0.05$). B) Influence of cotyledon orientation on the shoot regeneration efficiency.C)The highest regeneration efficiency was observed in abaxial orientation in contrast to adaxial orientation and were statistically significant ($p>0.05$). D) Regeneration efficiency between hypocotyl and cotyledon. (Values represented in percent mean ± SE).

rinsed three times with autoclaved double distilled water. The surface sterilized seeds wereimmersed in fresh 4% sodium hypochlorite solution for 10 min followed by washing with sterilized water for several times. Seeds were blot dried on sterilized tissue paper and were sown in seed germination medium (Table 1) in culture bottles. Culture bottles were kept initially for two days in dark at 25±1°C and later were exposed to photoperiod of 16/8 h in culture racks with an illuminationof light intensity 40-60µmol/sec.

Gene constructs preparation

Two *H.armigera*target genes serine protease (*HaSP*, 500 BP) and chymotrypsin (*HaCHY*, 465 BP) fragments wereselected. Both the gene fragments were PCR amplified usingprimers incorporating the restriction sites*Bam*HIin forward and *Sal*I inreverse primers.The RNAi assemblies for individual genes were prepared using pGRNAi vector (Manamohan *et al*., 2013),the gene constructs were prepared with *HaSP* and *HaCHY* genes and are designated as *pGRNAi-HaSP* and *pGRNAi-HaCHY*respectively. Both the binary vectors were electroporated into the *Agrobacteriumtumefaciens* strains EHA 105 containingpSOUPplasmid. The electroporated clones were selected in yeast extract mannitol (YEM) agar medium containing antibiotics, i.e.,10mg/lrifampicin, 10mg/ltetracyclineand 50mg/l kanamycin.

*Agrobacterium tumefaciens*strain

Agrobacterium strain EHA 105 was used for transformation, the cells were streaked and maintained in YEMmedium supplemented with 100 mg/l kanamycin. Single colony from the plate was picked in YEM broth with 100mg/l kanmycin and incubated at 28°C, once the culture attainsthe 0.1 OD_{600nm} and then it wasused for co-cultivation of the explants.

Explant preparation

Tomato seedlings of twelve day old were used for the preparation of explants, the top 5-6 mm highly regenerative, meristematicportion of the hypocotyl was removed and rest was used for the preparation of hypocotyl segments.The hypocotyl was cut and categorized into three segments, proximal, middle, distal and weredesignated as HYPseg-I, HYPseg-II and HYPseg-III respectively(Figure 1).All the hypocotyls segments were cut with the size of 0.8 to 1cm in length and were incubated separatelyonto the MS medium.For the cotyledon explant preparation, a small portion of cotyledons from both the end was excised and placed on the MS medium in both abaxial and adaxial orientation.

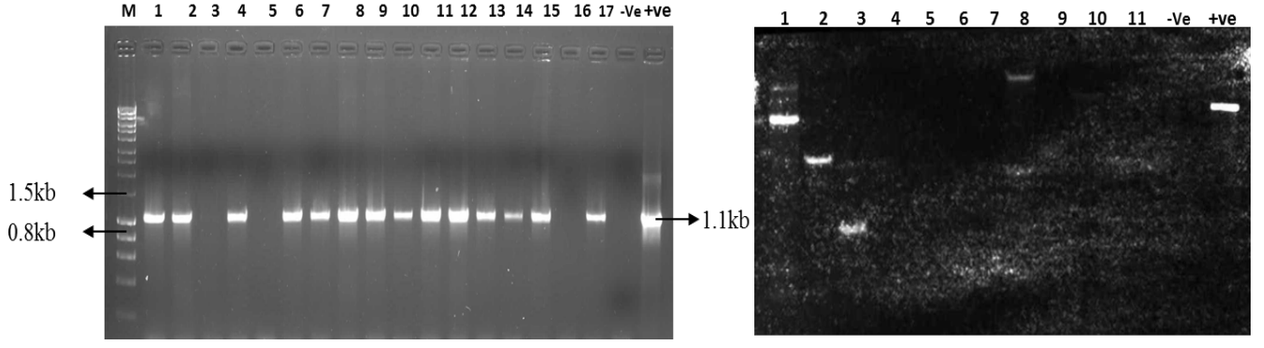

Figure 4. Molecular analysis of the primary putative transgenic plants. A) the PCR confirmation of the nptII gene M: Molecular Weigh Marker- DNA Hyper ladder-I (Bioline), sample nos 1, 2,4, 17and from 6 to 15 containing PCR product of 1.1 kb indicating the presence of the transgene in these plants, -ve : untransformed tomato plant. +ve : positive control (plasmid containing transgene). Figure 4B. Southern blot analysis of the of the selected putative transgenic plants, Lane no 1,2,3,8 and 11 hybridization signals were observed, -ve ; no hybridization signal in the wild type tomato plant. +ve ; plasmid with insert sample served positive control.

***Agrobacterium* mediated transformation**

The explants prepared were transferred to the preculture medium. Hypocotyl segments thuscategorized were placed separately on the MS medium, similarly, cotyledons were placed in adaxial andabaxial orientation, and the explants were incubatedfor two days. After that, the explants were cocultivated with *Agrobacterium* cell suspension for 10 min with 0.1 OD_{600nm}. The explants were blotted dry with sterile tissue towel and were shifted once again to fresh preculture medium for 48 hrs. After that, all the explants were transferred to the plates containing MS medium supplemented with augmentin and incubated for 48 hrs, to eliminate the *Agrobacterium*. The explants were finally shifted to the antibiotic selection medium contained kanamycin and augmentin. The explants were incubated in the antibiotic selection medium for 3 to 4 weeks, subsequentlysubcultured to the same fresh medium. The numbers of shoot regeneratedwere recorded for all the plates and used for calculating the shoot regeneration efficiency.The shoot regeneration efficiency was calculated by dividing the numbers of shoots regenerated to the total numbers of explant inoculated multiplied by hundred. The healthy, grown shoots were excised and shifted to the elongation medium, then to rooting medium. The well profusely rooted plants were acclimatized and maintained in the biosafety net house.

DNA Isolation and PCR screening

The genomic DNA was isolated from the young leaves of putative transgenic plants according to Lodhi*et al*. (1994). The isolated DNA was quantified using the Nanodrop™. For the PCR reaction 100 ng of template DNA, 10X buffer, 200 µM dNTP's, 2 units of TaqDNA polymerase (Genei, Bengalure) and 1µM of each primer was used for 25 µl PCR, the primer *npt*II forward '5 GATACATGAGAATTAAGGGAGTCAC3 and *nos* promoter reverse '5 TCAGAAGAACTCGTCAAGAAG 3' was used to amplify the chimeric region of *nos* promoter and the *npt*II region. The following reaction conditions were set for amplification, initial denaturation 95°C for 4 min, denaturation 94°C for 30 sec, annealing 54°C for 35 sec, extension at 72°C for 1 min and final extension was performed at 72°C for 10 min, the PCR products were resolved on 1.2% agarose TAE (Tris acetate EDTA) gel and documented.

S**tatistical analysis:** For each treatment five replicates with 25 numbers of explant were maintained, the analysis of the data were presented withstudent's 't' test utilizingGraphPadprism®softwere(USA).

Southern blot hybridization analysis

For the determination of copy number Southern hybridization wasperformed, a selected number of transgenic plants were analyzed, DNA was isolated utilizing theprocedure as described by Allen *et al*.(2006). About 10 µg of DNA was digested with *Bam*HI restriction enzyme and resolved on 0.7% agarose TAE gel. The separated DNA on the gel was transferred to positively charged nylon membrane using 20X SSC(saline sodium citrate).*npt*II probe of size 1.1 kb was labeled with Digoxigenin-11-dUTP alkali-labilereagent (Roche diagnostics, Germany) was used. The initial steps of Southern hybridization were followedfrom depurination to UV crosslinking according to the protocol described in (Sambrook and Russell, 2001) and stringency wash and detection steps were followed according to the manufacturer's instructions. For the detection of the hybridization signals CDP-Star and chemiluminescence detectorwas employed.

Results and Discussion

In the present investigation, *Agrobacterium* mediated transformation of tomato cultivar *Arkavikas*was used to determine the morphogenetic capability of hypocotyl segments. The shoot regeneration efficiency was determined and optimized for two widely utilized explants, hypocotyl and cotyledon.

The regeneration response of hypocotyl segments

In our previous experiment, we have observed that, use of hypocotyl explants resulted in higher regeneration rates than the cotyledons (Manamohan *et al*., 2011). In the present investigation, *Agrobacterium*

mediated transformation was carried to determine the morphogenetic potential among the hypocotyl segments. The hypocotyl explants wereprepared by dissecting it transversely into three segments(Figure 1) HYP-seg I (proximal), HYP-seg II(middle) and HYP-seg III(distal) and the regeneration from each of these groups was monitored.Interestingly, there was a significant difference in shoot regeneration response was observed among the hypocotyl segments. Overall, the *pGRNAi-HaSP* and *pGRNAi-HaCHY*transformedHYP-seg I showed similar and highest shoot regeneration percentage compared to other two segments (Figure 2A).

The regeneration percentage of HYP-seg-I obtained in both the construct was 28.65% and 23.23%. Similarly,in HYP-seg II transformed had 11.86% and 10.79%and for HYP-seg IIIwas 11.20%and 11.61% respectively, (Figure 3 Aand B). The overall regeneration response of HYP-seg I was significantly higher ($p>0.05$) than the other two segments, the HYP-seg IIand the HYP-segIII, which did not exhibit significant variations in regeneration response in independent experimentation.However, maximum rate of shoot regeneration was obtained in the Hyp-seg Iin both the vector transformed.

The response of hypocotyl in the induction medium was observed, in all the hypocotyl segment types regeneration response by forming calli was observed within 10 to 12 days and the appearance of shoot bud started in 14 to 24 days. In our experiment, we could obtain the highest regeneration percentage in hypocotyls, HYP-Seg-I had responded very well and yielded highest regeneration percentage in contrast to the other two hypocotyl segments. This may be due to the influence of the endogenous content and the polar transport of the phytohormone cytokinins and auxins and the polarity of the explant. The fate of explant organogenesis *in vitro* is decided by the amount of auxin to cytokinin ratio or balance between the endogeneous hormone concentration of the explant to the external supplemented hormones, variation in the endogenous hormone concentration in the explant yields a different regeneration response, i.e., it leads to a callus or shoot or root organogenesis. Parez-jimenez *et al*. (2014) reported the changes in the endogenous hormone concentration such as zeatin (Z), zeatinriboside (ZR), indole-3-acetic acid (IAA), abscisic acid (ABA), ethylene precursor 1-aminocyclopropane-1- carboxylic acid (ACC), salicylic acid (SA), and jasmonic acid (JA) when the explant was cultured with the external supply of growth hormone in the media. However, in the present study, we did not quantify the hormone composition of the both hypocotyl segments and cotyledon explants, possibly the quantification of the endogenous hormone concentration would give answer to the difference in the regeneration efficiency between the segments and explant types. Similar type of observation were documented in hypocotyls segments of *Capsicum*, where the proximal dissected part produced shoot buds, central part produced roots and distal parts produced more callus (Manamohan and Mythali, 2011). However, in our experiment on tomato, all the parts of the hypocotyl segments produced the shoots buds. The endogenous presence of the cytokinin in the hypocotyl varies with type of explant andwith plant species (Parez-jimenez *et al*., 2014; Coenen and Lomax, 1998). The changes in the expression level of genes encoding for hormone receptor also contributes the difference in the shoot regeneration efficiency (Wang *et al*., 2014; Motte *et al*., 2013; Close *et al*., 1989).The fate of the cell to undergo shoot or root organogenesis is controlled by expression of Regeneration1 (*Rg1*) as characterized in tomato cultivar microtom, *Rg1* increases the capacity of explant to form shoot or root in the medium (Lombardi-Crestena *et al*., 2012). The dissected segments of hypocotyls of tomato in the MS medium had showed differential morphogenetic capability, the proximal segment had more morphogenetic ability to form shoot buds than other progressively distant dissected segments, as a result, proximal segment had highest regeneration percentage than others. A number of authors reported the highest regeneration using hypocotyl in tomato (Shivankalyani *et al*., 2014; Yasmin. 2009; Gubies *et al*., 2003), however, this experiment further refined the use of proximal portion of hypocotyl segment to obtain the best regeneration response.

To our knowledge, this is the first report on the use of different hypocotyl segments to obtain higher regeneration and the superiority of HYPseg-I for highest shoot regeneration in the *Agrobacterium* mediated tomato transformation.

Regeneration response of cotyledon

The influence of orientation of cotyledon on shoot regeneration evaluated. The cotyledons were inoculated in both, abaxialand adaxial orientations on separate culture dishes containing MS medium supplemented with BAP 2mg/l and 0.1mg/l IAA in the antibiotic medium. The cotyledon inoculated with abaxial surfaces had given highest regeneration percentage (20.83%) as compared to adaxial surface (10.89%) (Figure 3C), the results were statistically significant ($p>0.05$). In the antibiotic selection medium, the cotyledon expanded in size and was green in color. The initiation of the callus during the firstweek and most of them completed callus formation by second week. The initiation of the shootbuds started at the cut end of the cotyledons on 10^{th} day and continued for 28 days. The cotyledons that wereplaced adaxially in the antibiotic selection medium were become curved and turned into cup shaped structures (Figure 2C), whereas the abaxiallyinoculated explants appeared dome shape in the center and some were completely flat in structure (Figure 2B). However, morphogenetic potential of the abaxially placed cotyledon was higher compared to adaxial orientation. A clumps of multiple shoot buds were appeared and were turned intoa rosette form, the well-elongated shoots were excised carefully and were shifted to elongation medium supplemented with GA_3 0.1mg/l. The cotyledonexplants that were not transformedwere turned gradually into brownish yellow in colour and such explants were discarded. Similar type of regeneration response was recorded by Bhatia *et al.* (2005), they studied the influence of the abxial and adxial cotyledon orientation and genotypic dependent regeneration in 10 tomato cultivars, they observed the highest regeneration in abaxial than in adaxial orientation. Rani *et al*. (2013) also reported the highest regeneration percentage in abaxial orientation in the two tomato cultivar Hisar Arun and Hisar Lalit, However, in contrast to our results Duzayman *et al*. (1994) obtained opposite results, but Costa *et al*. (2000) reported similar type of regeneration response in both the orientation. In Apple, the orientation of the cotyledon explant had positive influence on the regeneration, abaxial orientation of the cotyledon gave a highest regeneration percentage of 39.3 (Zhang *et al*., 2013). Hence, orientation of incubation plays a key role in regeneration.

The successful recovery of morenumber ofshootsand higher regeneration percentage in abaxially placed cotyledon in the antibiotic selection medium was due to the healthy nature of the explants and the contribution of the external hormonal supply, where as in adaxialorientation, due to the cup shaped structure of cotyledon sheltered the growth of *Agrobacterium* which inhibited the maximum shoot regeneration, where this *Agrobacterium* escaped during the blotting step after cocultivation. It was also reasoned that the cut ends of the cotyledon, which were facing upwardsfailed to absorb sufficient nutrients from the medium.The abaxially placed cotyledon explantswere well swollen in size in the media compared to adaxially placed cotyledon. A variation in the expression of Filamentous Flower (*FIL*) and the activity of the microRNA miR165/166 in abaxial side and are specific to the abaxial side (Tameshige *et al*., 2013). Although cotyledon contributed, more numbers of shoot buds but it was difficult to excise and they could not come out from the medium. All the shoots, which were obtained, were rooted in rooting medium containing 0.5mg/l indole-3-butyric acid (IBA) (Figure 2D) and were shifted to hardening facility, then to a biosafety net house facility for maintenance.

Regeneration of hypocotyl v/s cotyledon

The regeneration response was compared between hypocotyl and cotyledon, It was recorded highest in hypocotyl 25.76% and lowest in cotyledon 16.31% (Figure 3D) and were statistically significant. So far, a number of explants have been examined and reporteddiplomatic results on the success of type

of explants in highest shoot regeneration, this is probably due to the variation in the genotype. Some opinioned that cotyledon explants are superior (Ajenifujah-Soleboet *al*., 2012; Zhang *et al*., 2012; Bhatia *et al*., 2005) and some were hypocotyls in tomato regeneration(Shivankalyani*et al*., 2014; Yasmin,2009), but in our case hypocotyl weresuperior to cotyledon in terms ofshoot regeneration. Although the shoot bud formation of the cotyledons in the antibiotic selection medium was more but most of themwere failed to elongate into complete shoots. However, in hypocotylsegments such difficulties were not observed. Similar observations were recorded by Gubiš *et al*. (2003) who obtained highest regeneration in hypocotyl compared to cotyledon as he studied in13 tomato cultivars, Mathews *et al*. (2003) have also reported that consistency of high transformation rate using hypocotyl as explants of choice for regeneration usingtomato cultivar micro-tom andhypocotyls explants offered easiness for manipulation.Similarly, Yasmin, (2009) also reported that his choice of explants in terms of highest regeneration was hypocotyl.

Molecular characterization of the transgenic tomato plants:

All the putative T_0primary transgenic plants were PCR examined for the presence of insert using nos promoter forward and *npt*IIreverse primers. The transgenic plants amplified the 1.1 kb fragment and confirmed insert gene in the transgenic plant (Figure 4A).Plasmid DNA and DNA from control-untransformed plant were used as a positive and negative control respectively. Selected PCR confirmed plants were assessed for copy number. The Southern hybridization results indicated the single copy gene integration in the genome(Figure 4B).

Conclusion

The present investigation successfully demonstrated thatthe proximal segments ofhypocotyls as the best explant for the *Agrobacterium* mediated tomato transformationas they exhibited highest shoot regeneration among the hypocotyl explants as well as the coltyledons. This study also demonstrated the effect of orientation of incubation of cotyledons on the regeneration efficiency, where abxial incubation yielded higher regeneration than the adaxial incubation. The protocoldescribed hereis helps in obtaining rapid and efficient transformation of tomato to achieve higher throughputs.

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